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Nicotinic acetylcholine receptor contains multiple binding sites: Evidence from binding of α-dendrotoxin

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ABSTRACT **We have studied the stoichiometry of the binding of the long α-neurotoxins from the venom of *Dendroaspis viridis* (α-dendrotoxin) and *Naja naja siamensis* (α-cobratoxin) to the membrane-bound acetylcholine receptor (AcChoR) from *Torpedo californica* electric organ. The number of toxin molecules bound to one AcChoR molecule was determined by simultaneous-quantitative gas-phase microsequencing of all the amino acid sequences present in AcChoR–α-neurotoxin complexes. This method permits the use of homogeneous (nonradiolabeled) preparations of native toxins to obtain molar ratios of neurotoxin–receptor complexes. The stoichiometry obtained for α-cobratoxin was 2.1 ± 0.2 (n = 4), in agreement with the accepted view that α-cobratoxin, like α-bungarotoxin, binds to the two α subunits, which are constituent polypeptides of the AcChoR molecule. α-Dendrotoxin gave a stoichiometry of 4.1 ± 0.5 (n = 12); therefore, the AcChoR molecule contains four binding sites for this α-neurotoxin, two of which are recognized by α-cobratoxin. In support of this contention we have also found that when the AcChoR is saturated with α-bungarotoxin, addition of α-dendrotoxin markedly accelerates the dissociation of the bound α-bungarotoxin, demonstrating that the occupancy of the additional two sites by the latter toxin influences and decreases the affinity of the former toxin for its two binding sites. The fact that the AcChoR molecule is a pseudosymmetric complex of five highly homologous peptides suggests the possibility that as many as five binding sites for cholinergic ligand could be present, one on each subunit.**

The nicotinic acetylcholine receptor (AcChoR) from *Torpedo* electric organ is a complex pentameric molecule formed by four highly homologous proteins in a stoichiometry $\alpha_2\beta\gamma\delta$ (reviewed in ref. 1). The AcChoR structure is highly conserved along vertebrate evolution; nicotinic receptors from electric organs and muscle of distant animal species are consistently pseudosymmetric pentamers of four homologous subunits, and subunits of corresponding molecular weight from different AcChoRs are also highly homologous (2–11). Therefore, all the subunits of the AcChoR molecule studied so far are derived from a common ancestor, by means of gene duplications, which occurred very early during vertebrate evolution (2–4).

The AcChoR molecule contains a cation channel, whose opening and closing are controlled by the binding of cholinergic agonists and antagonists (reviewed in ref. 1). A major goal in elucidating the mechanism of AcChoR function has been to define the ligand binding events leading to activation of the ion channel. Both electrophysiological (12–15) and stopped-flow (16) studies indicated that the opening of the channel results from acetylcholine binding to two low-affinity sites, which operate somehow in a cooperative fashion. Ligand binding also causes the AcChoR to slowly become desensitized to a state characterized by inability of the channel to open and by high affinity for ligands (17–20). Since each of the two α subunits has a high-affinity binding site for cholinergic ligands (reviewed in ref. 1), several models of the linear type have been proposed in which both activation and desensitization of the receptor are controlled by ligand binding to these two sites and trigger physiological effects by sequential mechanisms involving multiple conformational changes with concomitant alterations in ligand affinity (reviewed in ref. 1). However, the relatively early divergence of the genes encoding the AcChoR subunits, as well as the extremely high degree of homology among corresponding subunits from distant animal species, suggests that the AcChoR subunits evolved separately to perform different functions (such as activation and desensitization) and that each subunit could in principle have an independent ligand binding site.

The long α-neurotoxins from snake venoms are a group of polypeptide cholinergic antagonists that are useful for study of the AcChoR binding sites, since they bind in a specific and very slowly reversible fashion (21). The only α-neurotoxins that have been extensively used are α-cobratoxin (α-Ntx) from the venom of *Naja naja* species and α-bungarotoxin (α-Btx) from *Bungarus multicinctus*. α-Ntx is widely used as a ligand for affinity chromatography to purify the AcChoR from various sources (reviewed in ref. 1). α-Btx has been shown to bind to the α subunits (22, 23), and since the binding of α-Btx and α-Ntx is mutually exclusive and they both inhibit photoaffinity labeling of the α-subunit (24, 25), it is believed that also α-Ntx binds to the high-affinity binding sites for cholinergic ligands present on the α subunits. Results, obtained with α-Ntx cross-linked to the AcChoR subunits and with an electron microscopic approach using α-Ntx complexed to biotin–avidin, support the notion that also α-Ntx binds to the α subunits (26, 27). α-Ntx and α-Btx bind to the AcChoR molecule with a stoichiometry of 2:1 (reviewed in ref. 1).

If binding sites for acetylcholine were to exist on all AcChoR subunits, one might expect that some other snake toxins could bind to some of the sites not recognized by α-Ntx and α-Btx, or to all of them. Such toxins would be valuable tools to determine the number and localization of the cholinergic binding sites present on subunits other than the α peptide.

Here we report studies of the binding stoichiometry of the long α-neurotoxin from *Dendroaspis viridis* (α-Dtx) and compare it with similar studies of α-Ntx. The binding of native toxins was measured by simultaneous-quantitative determination of the amino-terminal sequences present in the AcChoR–toxin complexes. We found that whereas four molecules of α-Dtx can complex with the AcChoR, only two

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Abbreviations: AcChoR, acetylcholine receptor; α-Dtx, α-dendrotoxin; α-Ntx, α-cobratoxin from *Naja naja siamensis*; α-Btx, α-bungarotoxin; ^{125}I-α-Btx, α-Btx radiolabeled with ^{125}I; BrAcCho, bromoacetylcholine.

molecules of α-Ntx can bind to one AcChoR molecule in a slowly reversible fashion. Therefore, the AcChoR molecule contains at least four binding sites for these cholinergic antagonists of which only two are recognized by α-Ntx and α-Btx. Ternary complexes between AcChoR and both α-Btx and α-Dtx are possible, as demonstrated by the increase of dissociation rate of ^{125}I-labeled α-Btx (^{125}I-α-Btx) bound to AcChoR when α-Dtx is present.

MATERIALS AND METHODS

α-Dtx and α-Ntx Purification. α-Dtx was purified from *Dendroaspis viridis* (Sigma) by the method of Shipolini *et al.* (28). α-Ntx was purified from *Naja naja siamensis* venom (Biotoxins, St. Cloud, FL) as described by Ong and Brady (29). The peptide composition of the column fractions was assessed by NaDodSO$_4$/polyacrylamide gel electrophoresis (30) using slab gels containing exponential or linear gradients of acrylamide from 15 to 22.5%. The identity and purity of isolated toxins was assessed by amino-terminal amino acid sequence (see below).

Protein Determination. Protein concentrations were measured by the method of Lowry *et al.* (31) or with the fluorescamine assay (32).

AcChoR Purification. Membrane-bound AcChoR was prepared from *T. californica* electric organs as described (33) and submitted to two cycles of alkali extraction (34, 35) to remove extrinsic membrane proteins. The concentration of α-Btx binding sites was measured by the DEAE-disk assay of Schmidt and Raftery (36) with ^{125}I-α-Btx (New England Nuclear). The specific activity of ^{125}I-α-Btx, isotopically diluted with α-Btx purified as described (29) was measured by the method of Blanchard *et al.* (37). NaDodSO$_4$/gel electrophoresis of AcChoR preparations was performed according to Laemmli (30), using gel slabs 1 mm thick and containing 8.75% acrylamide.

Preparations of purified membrane-bound AcChoR had specific activities ranging between 4 and 7.5 nmol of α-Btx binding per mg of protein. Upon NaDodSO$_4$/gel electrophoresis the four AcChoR subunits were the only major protein bands present. A protein of M_r ≈94,000 and components of M_r <30,000 (which are believed to be degradation products of the AcChoR subunits) were occasionally present.

Formation of Toxin/AcChoR Complexes. Aliquots of membrane-bound AcChoR in 10 mM sodium phosphate buffer, pH 7.0, were incubated for 1 hr at 60°C with a 5- to 10-fold excess of αDtx or α-Ntx. The AcChoR concentration was determined, following Triton X-100 solubilization, from the binding of ^{125}I-α-Btx, assuming that two α-Btx molecules bound to one AcChoR molecule under these conditions. The membrane fragments were washed and centrifuged twice in a Beckman Microfuge at 6°C for 15 min using ice cold 50 mM ammonium bicarbonate at pH 9. Preliminary experiments demonstrated that this volatile buffer did not influence the dissociation rate of α-Btx–AcChoR complexes. The washed membrane-bound AcChoR–toxin complexes were solubilized with 1.5% NaDodSO$_4$ in 50 mM ammonium bicarbonate and lyophilized several times to eliminate the buffer. The NaDodSO$_4$ had been recrystallized twice from hot ethanol.

Amino-Terminal Amino Acid Sequence Analysis. The lyophilized AcChoR–toxin complexes with NaDodSO$_4$ were dissolved in glass-distilled water and submitted to amino-terminal amino acid sequencing by automated Edman degradation in a gas-phase sequencer (Applied Biosystems, Foster City, CA). Phenylthiohydantoin-derivatized amino acids were identified by HPLC on an IBM Cyano column (38).

Effect of α-Dtx on the Dissociation Rate of α-Btx–AcChoR Complexes. ^{125}I-α-Btx–AcChoR complexes were formed by incubating membrane bound AcChoR (1–14 μM in 10 mM phosphate buffer, pH 7.0) for 1 hr at 6°C with a 2-fold excess of radiolabeled toxin. The unreacted toxin was washed away by diluting the reaction mixture with 30 ml of the same buffer and pelleting the membrane fragments at 19,000 rpm for 20 min in a Sorvall SS-34 rotor. The washed complexes were resuspended in a minimal volume (≈300 μl) of the same buffer, and aliquots of 0.1–0.8 nmol of AcChoR (measured as α-Btx binding sites) were incubated at 60°C with a 10-fold excess of either unlabeled α-Btx or α-Dtx. The final AcChoR concentration was 1 μM. Aliquots of the mixtures were taken at time intervals and pipetted onto DEAE disks. The disks were washed as described (36) and the ^{125}I-α-Btx still bound to the AcChoR was determined using a Beckman gamma counter. Previous experiments (not reported here) showed that membrane-bound AcChoR binds quantitatively to DEAE disks. The dissociation rate of the α-Btx–AcChoR complexes was calculated from the amount of radioactive toxin still bound to AcChoR at the various time intervals, using an exponential fitting program.

RESULTS

Purification of α-Dtx and α-Ntx. Different toxins capable of interference with cholinergic transmission can be isolated from *D. viridis* venom. The elution profiles we obtained in these studies for the three chromatographic steps of the purification procedure were in excellent agreement with those reported (28). The final α-Dtx peak was desalted, lyophilized, and submitted to amino-terminal amino acid sequencing. The sequence obtained corresponded to that of α-Dtx [toxin 4.9.3 of Banks *et al.* (28, 39)]. Marginal amounts (<4%) of the short α-neurotoxin designated by Banks *et al.* (39) as toxin 4.9.6 were consistently present in α-Dtx preparations. This toxin can bind to AcChoR (data not shown), and since its sequence is known (40), this was taken into consideration in the analysis of the sequences present in the α-Dtx–AcChoR complexes. Purified α-Ntx preparations were consistently found to be free of any contaminating material by sequence analysis, which yielded only the known amino acid sequence (21).

Determination of the Stoichiometry of α-Dtx and α-Ntx Binding to AcChoR. This was achieved by simultaneous quantitative determination of the amino acid sequences present in washed toxin–AcChoR complexes. In Fig. 1 the amino-terminal sequences of the four AcChoR subunits are compared with the corresponding segments of α-Dtx (*A*) and α-Ntx (*B*). For quantitation of multiple sequences, careful evaluation of the repetitive yield of the Edman degradation

A

	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
α	S	E	H	E	T	R	L	V	A	N	L	L	E	N	Y
β	S	V	M	E	D	T	L	L	S	V	L	F	E	T	Y
γ	E	N	E	E	G	R	L	I	E	K	L	L	G	G	Y
δ	V	N	E	E	E	R	L	I	N	D	L	L	I	V	N
α-Dtx	R	T	C	Y	K	T	P	S	V	K	P	E	T	C	P

B

	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5
α	S	E	H	E	T	R	L	V	A	N	L	L	E	N	Y
β	S	V	M	E	D	T	L	L	S	V	L	F	E	T	Y
γ	E	N	E	E	G	R	L	I	E	K	L	L	G	G	Y
δ	V	N	E	E	E	R	L	I	N	D	L	L	I	V	N
α-Ntx	I	R	C	F	I	T	P	D	V	T	S	K	D	C	P

FIG. 1. Amino-terminal amino acid sequences of the four AcChoR subunits α, β, γ, and δ (from ref. 2) and of α-Dtx (*A*) and α-Ntx (*B*). The sequences of the toxins are from refs. 39 and 21.

during each sequence determination is essential, and this can be obtained from the sequences of the AcChoR subunits, by comparing (using the single-letter notation for each amino acid residue with a subscript to designate the position of that residue from the amino terminus) V_1 (δ) with 1/2 of V_8 (α_2), 1/2 of N_2 (γ, δ) with N_9 (δ) and L_7 (α_2, β, γ, δ) with L_{11} (α_2, β, γ, δ). In the case of α-Dtx, the binding stoichiometry can be calculated best by comparing at least two of the following residues: R_1 with 1/4 of R_6 (α_2, γ, δ); T_2 with 1/2 of T_5 (α_2); Y_4 with 1/5 of E_4 (α_2, β, γ, δ); Y_4 with M_3 (β), D_5 (β), 1/5 of L_7 (α_2, β, γ, δ) and 1/4 of V_8 + I_8 (α_2, γ, δ). When residues from different cycles are compared, they must be normalized using the repetitive yield of that particular sequence run, calculated as described above. The values obtained were averaged to obtain the final α-Dtx/AcChoR stoichiometry. The α-Ntx/AcChoR stoichiometry was obtained in a similar fashion by comparing the following residues: I_1 with V_1 (δ); R_2 with 1/4 of R_6 (α_2, γ, δ); I_1 and I_5 with 1/2 of I_8 (γ, δ); F_4 with 1/5 of E_4 (α_2, β, γ, δ) and with F_{12} (β); F_4 and I_5 with 1/5 of L_7 (α_2, β, γ, δ).

The stoichiometry values were obtained for α-Dtx and α-Ntx in different experiments, using different AcChoR and toxin preparations. The average value obtained in the presence of saturating amounts of toxins (molar ratio 5–80:1) was 4.1 ± 0.5 (n = 12) for α-Dtx and 2.1 ± 0.2 (n = 4) for α-Ntx. When subsaturating amounts of α-Dtx were used, the amount of bound α-Dtx increased linearly and reached a plateau of approximately four molecules of α-Dtx bound per AcChoR molecule at a toxin/AcChoR concentration of 4:1 (AcChoR, 2 μM; α-Dtx, 8 μM) (Fig. 2).

Effect of α-Dtx on the Dissociation Rate of α-Btx–AcChoR Complexes. The basal dissociation rate of the complexes between ^{125}I-α-Btx and AcChoR was measured in the presence of an excess of unlabeled α-Btx for incubation periods of up to 48 hr. The half-life of the complexes was found to be >250 hr, in good agreement with the accepted notion that this toxin forms very slowly reversible complexes (37). When unlabeled α-Dtx was present, the dissociation rate was markedly enhanced, and the half-life was reduced to 6.15 ± 2.13 hr (n = 6) (Fig. 3).

DISCUSSION

A major goal in the elucidation of AcChoR function is to define the events mediated by ligand binding that lead to channel activation and desensitization. For this purpose, it is of paramount importance to define the number, location, and function of all binding sites for cholinergic ligands. Here we directly demonstrate the existence of four binding sites on the AcChoR molecule, obtained from the binding of α-Dtx, an irreversible cholinergic ligand.

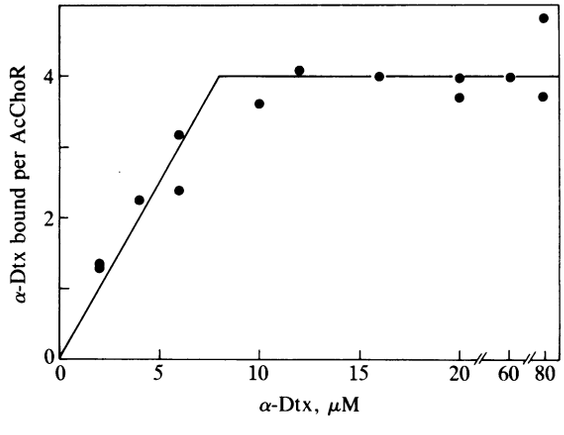

FIG. 2. Concentration dependency of α-Dtx binding to membrane-bound AcChoR. The amount of bound α-Dtx to one AcChoR molecule increases linearly with the toxin concentration until it reaches saturation when four α-Dtx molecules are bound, when the α-Dtx concentration is about four times the AcChoR concentration (2 μM).

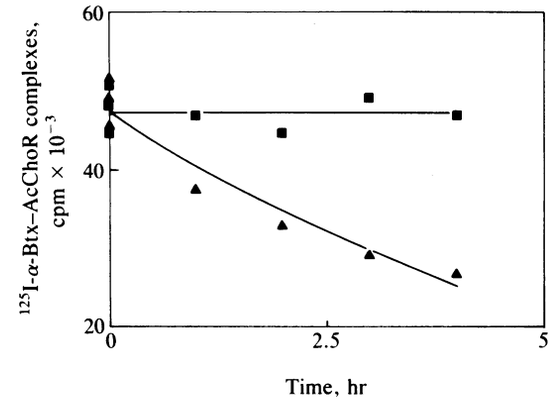

FIG. 3. Effect of the presence of unlabeled α-Dtx (▲) or α-Btx (■) on the dissociation rate of complexes formed between ^{125}I-α-Btx and membrane-bound AcChoR. The basal rate of dissociation of ^{125}I-α-Btx is very slow, with a half-life >250 hr. When α-Dtx is present, the dissociation rate is markedly enhanced (half-life in this experiment, 4.4 hr; correlation coefficient, 0.973).

It has generally been assumed that activation and desensitization are controlled by two quasi-identical binding sites located on the two α subunits present in the AcChoR molecule (reviewed in refs. 1 and 16). This assumption was based on the facts that (*i*) the α subunits can be labeled with high affinity by cholinergic affinity reagents, such as (maleimidobenzyl)trimethyl ammonium chloride (24, 41) and bromoacetylcholine (BrAcCho) (25, 42–44) by virtue of the existence of a readily reducible disulfide bond near the site, and (*ii*) the α subunit binds α-Btx (22, 23). Many attempts have been made to compare the *in vitro* binding of cholinergic ligands with the affinity and rate characteristics predicted for the activating binding site from electrophysiological (12–15) and stopped-flow (16) experiments. A major discrepancy was consistently found between the apparent dissociation constant measured by direct ligand binding *in vitro* and those characterizing the permeability response (reviewed in ref. 1). This led to proposals of complex ligand binding mechanisms, involving sequential transitions of affinity states (reviewed in refs. 1 and 16).

The demonstration that all the peripheral receptors studied so far are pseudosymmetric pentamers of highly homologous subunits (2–4, 11) supports the notion that homologous binding domains could exist on some or possibly all the subunits. An indication in favor of this possibility was the observed labeling of more than one subunit by cholinergic affinity reagents (antagonists) that do not depend on disulfide bound reduction (45, 46). Furthermore, a low-affinity site(s) for agonists that is distinct from the well-documented high-affinity sites on the α subunit has been demonstrated by spectroscopic methods (47–49). The equilibrium and kinetic properties of the low-affinity site(s) are those expressed for a site involved in channel opening (47) and their properties are not influenced by AcChoR desensitization (48). Cholinergic agonists can still bind to these low-affinity sites with the same characteristics of affinity and rate after the two α subunits have been covalently labeled by BrAcCho (49). This suggests that the high-affinity sites on the α subunits are involved only or preferentially in inactivation processes such as desensitization. It is, therefore, crucial to measure by some direct method the number of binding sites for cholinergic ligands present on the AcChoR molecule. This question

cannot be answered by equilibrium binding studies or by the use of affinity ligands because stoichiometric binding to the low-affinity sites (K_d for acetylcholine ≈75 μM, K_d for carbamoylcholine ≈1 mM) would require the use of high concentration of radioactive ligand, causing major background problems. In addition, most of the affinity labels so far used require the presence of a disulfide bond in close proximity to the binding site, and other results (50) seem to indicate that the affinity-label (maleimidobenzyl)trimethyl ammonium chloride can bind to cysteine residues that are present only in the α subunit (51).

The α-neurotoxins are good ligands to study the binding-site stoichiometry, since they form very slowly reversible complexes with the AcChoR. Because of their hydrophilic nature the unbound toxin can be efficiently washed away resulting in minimal nonspecific binding. α-Dtx is a cholinergic antagonist closely related to α-Ntx and α-Btx because it belongs to the same protein family and is highly homologous to them (21, 39). These toxins are powerful blockers of muscle and electric organ AcChoR by virtue of their ability to bind to the two α subunits (22, 23, 26, 27) in a competitive fashion with acetylcholine, carbamoylcholine, curare, and other classic cholinergic ligands (21, 52). α-Btx binds to a segment of the α subunit very close to or overlapping with the segment containing a cysteine residue labeled by cholinergic labels (53).

α-Dtx and α-Ntx are peptide toxins 72 residues long. The sequence of the first 15 residues is reported in Fig. 1*A* and *B*. They both have residues that allow good quantitative comparison either with identical residues in the AcChoR sequence or with residues known to give a similar yield in the Edman degradation. Determination of toxin–AcChoR stoichiometry by simultaneous-quantitative sequencing of all the proteins present in the washed complexes has the following three major advantages in comparison with methods that utilize radiolabeled derivatives of the toxin: (*i*) it permits the use of homogeneous preparations of native toxin, never exposed to the rather harsh oxidizing conditions used for radiolabeling; (*ii*) it directly measures the amount of each peptide and their ratios and eliminates the need of determining protein content and specific activity of radiolabeled toxins, which are seldom highly accurate; (*iii*) the amounts of the different peptides are measured at multiple steps in the sequence, yielding multiple-independent determinations of the peptide stoichiometry of each toxin–AcChoR complex. Using radiolabeled derivatives of α-Ntx and α-Btx, these toxins have been found to bind to the AcChoR up to a maximum of 8 nmol of toxin per mg of pure AcChoR protein (reviewed in ref. 1). Since the M_r of the toxin is ≈8000 (21) and that of AcChoR is ≈270,000 (51), this corresponds to a stoichiometry of two toxin molecules bound to one AcChoR molecule, as expected for toxins that bind to the α subunits. In excellent agreement with these reports, we directly measured a binding stoichiometry for the native α-Ntx of 2.1 ± 0.2.

In spite of the considerable sequence homology with other snake α-neurotoxins (39), α-Dtx contains two amino acid substitutions at positions invariant in other α-neurotoxins. A glutamic acid replaces an invariant lysine at position 29, and a second glutamic acid occurs at position 43, which is always uncharged in the other α-neurotoxins (i.e., valine, isoleucine, or threonine). This region of the molecule (residues 24–39) is believed to be essential for function (54–56), these substitutions, involving a change of three in the net charge, might be expected to cause different binding properties. In agreement with this prediction, when the same approach used for α-Ntx was applied for measurement of the number of binding sites recognized by α-Dtx, a stoichiometry of 4:1 was found. Since α-Dtx can fully inhibit the binding of α-Btx to AcChoR (57), this means that α-Dtx can recognize two extra sites in addition to those on the α subunits. This point is further proven by the ability of α-Dtx to greatly accelerate the dissociation of bound ^{125}I-α-Btx from the AcChoR (Fig. 3). This effect is readily explained if α-Dtx can still bind to AcChoR saturated with α-Btx and form ternary complexes. As a result of α-Dtx binding to the sites not recognized by α-Btx, a conformational change must occur that modifies the affinity characteristics of the binding sites to which α-Btx is bound, and α-Btx is more quickly released.

Since α-Ntx and α-Btx bind to the α subunits, the other two cholinergic sites recognized by α-Dtx could be on any of the other three peptide components of the AcChoR. The fact that all the AcChoR subunits are highly homologous raises the possibility that as many as five potential binding sites for cholinergic ligands could exist, one on each subunit. A fifth site could either be unrecognized by α-Dtx or bind with lower affinity in a reversible fashion so that α-Dtx could dissociate during the washing procedures used.

It is conceivable that α-Btx and α-Ntx could also bind in reversible fashion to low-affinity sites other than those on the α subunits and that such binding cannot be detected by the conventional assays devised for measuring slowly reversible binding. In support of this are the following findings: (*i*) using affinity derivatives of α-Btx and α-Ntx, other subunits were labeled in addition to α, although to a lesser extent (26, 58, 59); (*ii*) in the brain low-affinity sites for α-Ntx and α-Btx have been demonstrated, in addition to the well-documented high-affinity sites and in equivalent number (60). The binding to these low-affinity sites consistently impairs cholinergic function (61, 62) whereas the binding to the high-affinity sites is generally functional but silent. α-Dtx blocks AcChoR function in neuronal systems that are not sensitive to α-Btx blockade, such as the frog spinal cord (63) and the snail neurons (64). In addition, the complexes that α-Dtx forms with neuronal AcChoR are more stable than those formed by α-Btx (57). These findings support the notion of a broader binding specificity of α-Dtx, which would, therefore, recognize and bind with high affinity to sites that α-Ntx and α-Btx bind with much lower affinity or not at all. In support of this contention, α-Dtx has been demonstrated to bind to twice as many sites as α-Btx in the sympathetic cell line PC12 (57). Since the high-affinity binding of α-Ntx and α-Btx to neurons is in general functionally silent (reviewed in ref. 65), persistent doubts were raised as to whether the α-Btx binding component was, in fact, an AcChoR. These doubts were solved by the demonstration (65) that the α-Btx binding component(s) isolated from different areas of avian brain are multisubunit-complex proteins at least partially homologous to peripheral AcChoRs and carrying high-affinity sites for BrAcCho. Interestingly, the high-affinity site for BrAcCho is located on a subunit different from the one which, by molecular weight and sequence homology, was identified as α. The BrAcCho binding component can be cross-linked by α-Btx by using dimethysuberimidate (66) and has a M_r of 56,000, similar to the peripheral β subunits to which it could be homologous. These findings raise the interesting possibility that brain AcChoR works by a mechanism somehow different from peripheral AcChoR, involving multiple binding sites but with corresponding sites located on different subunits.

From all the above considerations, muscle and neuronal AcChoRs appear to be as complex proteins carrying multiple homologous domains and multiple binding sites for cholinergic ligands, possibly one for each subunit. All these sites could bind agonists and antagonists, but with a spectrum of affinities and functions different in different AcChoRs. At low-ligand concentration only high-affinity sites are revealed, both in terms of their number and of their effect(s). Only using special ligands with broad specificity, such as α-Dtx, or suitably high concentrations of the other ligands can one

reveal and study the low-affinity sites that are associated with channel activation and, therefore, normal physiological function.

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